

Nasal Immunization of Mice with Human Papillomavirus Type 16 Virus-Like Particles Elicits Neutralizing Antibodies in Mucosal Secretions

CAROLE BALMELLI,¹ RICHARD RODEN,² ALEXANDRA POTTS,¹ JOHN SCHILLER,²
PIERRE DE GRANDI,¹ AND DENISE NARDELLI-HAEFLIGER^{1*}

*Department of Gynecology, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland,¹ and
Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, Maryland 20892-4040²*

Received 22 April 1998/Accepted 24 June 1998

To specifically induce a mucosal antibody response to purified human papillomavirus type 16 (HPV16) virus-like particles (VLP), we immunized female BALB/c mice orally, intranasally, and/or parenterally and evaluated cholera toxin (CT) as a mucosal adjuvant. Anti-HPV16 VLP immunoglobulin G (IgG) and IgA titers in serum, saliva, and genital secretions were measured by enzyme-linked immunosorbent assay (ELISA). Systemic immunizations alone induced HPV16 VLP-specific IgG in serum and, to a lesser extent, in genital secretions but no secretory IgA. Oral immunization, even in the presence of CT, was inefficient. However, three nasal immunizations with 5 μ g of VLP given at weekly intervals to anesthetized mice induced high ($>10^4$) and long-lasting (>15 weeks) titers of anti-HPV16 VLP antibodies in all samples, including IgA and IgG in saliva and genital secretions. CT enhanced the VLP-specific antibody response 10-fold in serum and to a lesser extent in saliva and genital secretions. Nasal immunization of conscious mice compared to anesthetized mice was inefficient and correlated with the absence of uptake of a marker into the lung. However, a 1- μ g VLP systemic priming followed by two 5- μ g VLP intranasal boosts in conscious mice induced both HPV16 VLP-specific IgG and IgA in secretions, although the titers were lower than in anesthetized mice given three intranasal immunizations. Antibodies in serum, saliva, and genital secretions of immunized mice were strongly neutralizing in vitro (50% neutralization with ELISA titers of 65 to 125). The mucosal and systemic/mucosal HPV16 VLP immunization protocols that induced significant titers of neutralizing IgG and secretory IgA in mucosal secretions in mice may be relevant to genital HPV VLP-based human vaccine trials.

The “high-risk” human papillomavirus (HPV) types, most commonly type 16 (HPV16), are etiologically linked to over 90% of cervical cancers (7). Cervical cancer is the second leading cause of cancer deaths in women worldwide, encouraging the development of a prophylactic vaccine to prevent genital infection by these viruses. Vaccine development has been hindered by the difficulty of virus propagation in culture and the lack of animal models for the genital mucosotropic HPV type (34). However, expression of the papillomavirus major capsid protein L1 in mammalian, insect, yeast, or bacterial cells has been shown to generate virus-like particles (VLPs) (22, 24, 28, 30, 43, 51, 53). Parenteral injection of these VLPs elicits high titers of neutralizing antibodies in serum and protection from experimental challenge with infectious virus in animal papillomavirus models (10, 28, 29, 51, 53). Protection from experimental infection with cottontail rabbit papillomavirus and canine oral papillomavirus by passive transfer of immunoglobulin G (IgG) from immunized to naive animals has been demonstrated for rabbits (10) and dogs (56), respectively, indicating that cell-mediated effector immune responses are not required for protection.

Neutralizing antibodies must be present at the genital mucosal site of infection to completely prevent cervical HPV infection. Antibodies both pass from plasma into genital secretions and are synthesized by local plasma cells (48, 58, 64). The plasma cell precursors that migrate to the genital tract are

derived primarily from mucosal lymphoid tissues and mostly secrete IgA (9, 39). Stimulation of these cells requires that antigens have access to mucosa-associated lymphoid tissue (MALT). In several experimental systems, nasal instillation was the most effective route of immunization to generate specific antibodies in genital secretions in mice (15, 16, 20, 25, 27, 43, 47, 55) and in monkeys (52).

Systemic immunization of mice with purified HPV VLPs did not induce detectable genital mucosal antibodies (21), while low-titer VLP-specific IgG, but not IgA, was detected in cervicovaginal washes of parenterally immunized monkeys (33). The experiments in monkeys, however, showed that the transudated IgG alone partially and transiently neutralized HPV11 in vitro, suggesting that a local, sustained production of secretory IgA (sIgA) and/or specific IgG may be required for long-lasting sterilizing immunity. In this study, we have compared different protocols of immunization of mice, including nasal and oral mucosal routes, by using HPV16 VLPs purified from insect cells for their ability to induce HPV16 VLP-specific antibodies in serum and mucosal secretions of mice. Furthermore, the in vitro neutralizing activity of salivary and genital secretions containing specific IgA and/or IgG, and sera were compared by using an HPV16 pseudovirion neutralization assay (50).

MATERIALS AND METHODS

Purification of HPV16 VLPs, immunization, and sampling of mice. Baculovirus-derived HPV16 VLPs were purified as described previously (28) and diluted to a final inoculum volume of 20 μ l with either phosphate-buffered saline (PBS) for subcutaneous immunizations or PBS–0.5 M NaCl either alone or mixed with 5 μ g of cholera toxin (CT; Sigma) for nasal immunizations. Six-week-old female BALB/c mice were used in all the experiments. For oral immuniza-

* Corresponding author. Mailing address: Département de Gynécologie, c/o Institut de Microbiologie, Bugnon 44, 1011 Lausanne, Switzerland. Phone: 021/314 40 81. Fax: 021/314 40 95. E-mail: DNARDELL@ulrec1.unil.ch.

Nasal immunization under anesthesia

- 3 x 5 μ g HPV16 VLPs + 5 μ g CT
 ■ 3 x 5 μ g HPV16 VLPs

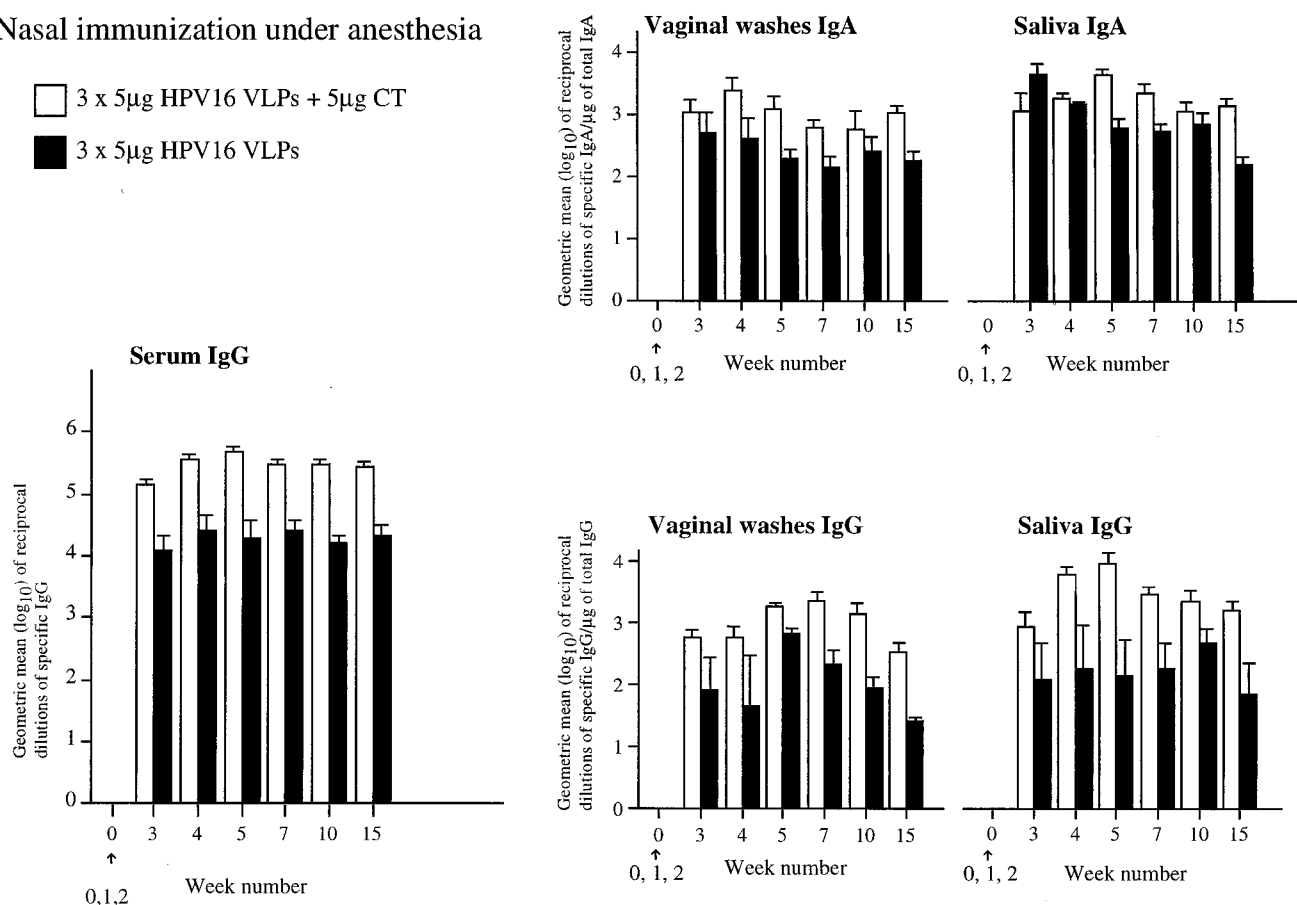


FIG. 1. Anti-HPV16 VLP systemic and mucosal antibody responses after intranasal immunization with purified HPV16 VLP in the presence or absence of the CT adjuvant. Two groups of four 6-week-old BALB/c anesthetized mice were immunized three times weekly with 5 μ g of HPV16 VLP alone or mixed with 5 μ g of CT by the intranasal route. Data are expressed as the geometric means (\log_{10}) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or specific IgG per microgram of total IgG in secretions. Error bars indicate the standard errors of the means.

tion, the mice were fed 30 μ l of 10% sodium bicarbonate to neutralize stomach acidity 5 min prior to administration of the 20- μ l inoculum. For nasal immunization, the mice were either conscious or anesthetized by intraperitoneal injection with 100 μ l each of both 0.2% xylazine hydrochloride (Rompun; Bayer) in PBS and 10 μ g of ketamine hydrochloride per ml (Ketavet; Parke-Davis) per 10 g of body weight. The inoculum (20 μ l) was introduced dropwise into one nostril. Subcutaneous immunization was performed at the base of the tail. Blood, saliva, and genital samples were taken as described previously (25). All samples were stored at -70°C . For neutralization experiments (see below), the saliva and genital washes were sterilized by irradiation (3,000 rads).

ELISA. The amounts of total IgA and IgG as well as anti-HPV16 VLPs antibodies were determined by enzyme-linked immunosorbent assay (ELISA) with biotinylated goat anti-mouse IgA (Kirkegaard & Perry Laboratories) or IgG (Amersham), respectively, as described previously (25, 43). End-point dilution of all samples was carried out. The specific IgA or IgG titers were expressed as the reciprocal of the highest dilutions that yielded an optical density at 492 nm four times that of preimmune samples. These reciprocal dilutions were normalized to the amount of total IgA or IgG in saliva and vaginal washes (25).

In vitro HPV16 neutralization assay. Infectious pseudotyped virions consisting of the HPV16 capsid, comprising L1 and L2, surrounding the bovine papilloma-virus type 1 genome, designated HPV16 {BPV1}, were generated as previously described (50). Infectious pseudotype HPV16 in cell extracts was quantitated by the induction of transformed foci in monolayers of mouse C127 cells. Neutralizing activity was measured after preincubation of the cell extracts with samples from mice at the indicated dilutions (final volume, 1 ml) for 1 h on ice. Preimmune samples and samples from mice immunized with an unrelated antigen (a recombinant *Salmonella typhimurium* strain expressing the nucleocapsid of hepatitis B virus: HBC antigen [25, 43]) were used as negative controls. Mouse monoclonal antibody H16.V5 raised against HPV16 VLP (a gift of N. Christensen, Milton S. Hershey Medical Center, Hershey, Pa.) was used as a positive control.

RESULTS

Intranasal immunization with purified HPV16 VLPs induces systemic and mucosal antibody responses. Groups of four anesthetized mice were immunized intranasally three times at weekly intervals with 5 μ g of purified VLPs, either with or without 5 μ g of CT, in a final volume of 20 μ l. Serum, saliva, and genital washes were taken 3, 4, 5, 7, 10, and 15 weeks after the first immunization. Both methods induced sustained high HPV16 VLP-specific antibody titers in serum and in genital and oral secretions (Fig. 1). The addition of CT resulted in a 10-fold increase in the specific IgG titers in the serum, while the effect on specific antibody titers in both saliva and vaginal washes was generally smaller. Overall, the titers of antibody were quite stable during the 15-week study. In contrast, oral immunization of mice with the same amount of VLPs did not induce VLP-specific antibodies in either serum or secretions whereas the addition of CT induced very low titers of VLP-specific IgG in sera and IgA in secretions (mean titers of 50 and 10, respectively; data not shown). Because the significant toxicity of CT precludes its use in humans and because of the highly immunogenic nature of VLPs, CT was not used in the subsequent experiments.

Effective intranasal immunization requires anesthesia. We had previously observed that the titers of antibodies generated

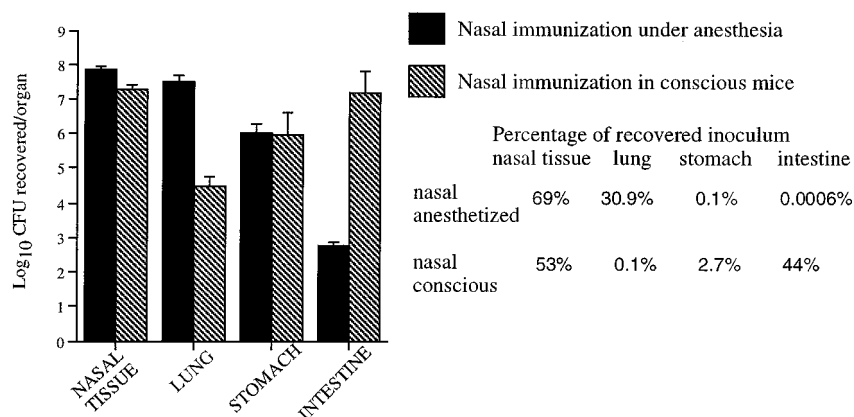


FIG. 2. Fate of an inoculum 15 min after intranasal immunization of anesthetized or conscious mice. Two groups of three 6-week-old BALB/c mice were immunized intranasally with 10^8 CFU of *S. typhimurium* PhoP^c in 20 μ l of PBS (25, 43) while they were under anesthesia or conscious. At 15 min later, the nasal tissue, the trachea and lungs ("lung"), the esophagus, pharynx, and stomach ("stomach"), and the intestine and rectum ("intestine") were recovered and homogenized in a Dounce homogenizer in 2 to 4 ml of 15% sucrose in PBS (43). Data are expressed as the geometric mean (\log_{10}) of recovered CFU per organ. Error bars indicate the standard errors of the means. The percentages of the recovered inoculum in each organ is also indicated.

by intranasal immunization with recombinant *Salmonella* varied between conscious and anesthetized mice (25), so we tested intranasal immunization with purified VLPs in conscious mice. The schedule of immunization and sampling was similar to that in the first experiment (Fig. 1). In contrast to mice immunized under anesthesia, mice immunized while conscious generated only very low titers of specific antibody titers in serum and in saliva (mean titers of 50 and 10, respectively) and no detectable antibody in vaginal washes (data not shown). To examine the role of anesthesia in the induction of anti-VLP antibodies after nasal immunization, we compared the fate of a live *Salmonella typhimurium* inoculum administered intranasally to conscious and anesthetized mice. The number of bacteria recovered in various organs was assayed to trace the inoculum (Fig. 2). The fate of the inoculum 15 min after immunization was quite different in conscious and anesthetized mice: a high percentage of inoculum (30%) was recovered from the lungs of mice immunized under anesthesia, but the mice immunized while conscious had a very small amount of inoculum in the lungs (0.1%). Instead, much of the intranasal inoculum (45%) in conscious mice was rapidly swallowed and found in the intestine. Similar portions of inoculum remained in the nasal cavity 15 min postimmunization in both protocols. These data suggest that the strong antibody response induced by nasal immunization with purified HPV16 VLPs of mice under anesthesia results from deposition of the antigen in the lungs.

The efficacy of nasal immunization is dose and regimen dependent. The HPV16 VLP-specific antibody responses were compared in mice immunized intranasally under anesthesia with a dose of either 5 or 1 μ g of purified HPV16 VLPs (Fig. 3) and a booster immunization with the same dose 8 weeks later. Samples of serum, saliva, and vaginal washes were taken 3, 4, 6, and 8 weeks after the first immunization and 2, 5, and 11 weeks after the booster dose. A single 1- μ g VLP dose induced only barely detectable titers of specific IgG in serum and specific IgA in saliva, and the booster immunization had no effect, whereas a single 5- μ g VLP dose induced low specific IgG titers in serum and genital secretions and a 10-fold increase in all specific antibody titers after the booster immunization. Although the specific IgG titers in serum after two 5- μ g VLP immunizations were similar to those measured with the three weekly 5- μ g doses (Fig. 1), the titers of specific antibodies

in secretions were lower and less stable over time after only two more widely spaced immunizations.

Systemic priming influences the mucosal response induced by nasal immunization. Although systemic immunization alone fails to induce sIgA in mucosal secretions, it has been previously shown to enhance the outcome of associated mucosal immunizations (18, 32, 38, 57). Moreover, systemic immunization is usually more effective at inducing specific systemic antibodies than are immunizations by mucosal routes, and the serum-specific IgG concentration can influence the amount of specific IgG transudating into genital secretions. We have therefore evaluated different combinations of systemic, i.e., subcutaneous, and intranasal immunizations. The effects of systemic boosting are depicted in Fig. 4. Mice immunized previously with three weekly intranasal 5- μ g doses (Fig. 1 and conscious mice) were given booster doses of 1 μ g of HPV16 VLP subcutaneously and samples of serum and secretions were taken 2, 6, and 12 weeks later (weeks 17, 21, and 27, respectively, in Fig. 4). A sharp but transient increase in specific IgG titers was observed in serum and secretions of all animals, including the nonresponder mice that had been immunized intranasally while conscious, thus indicating that those mice had not been tolerized. The systemic boost had no effect on the IgA titers in secretions and, at best, transiently boosted mucosal IgG titers (Fig. 4).

To analyze the effect of systemic priming followed by nasal boosting, 16 mice were subcutaneously immunized with 1 μ g of HPV16 VLPs, divided into four groups of four mice, and subsequently immunized at weeks 2 and 10 by different methods. The first and second groups were given two intranasal booster doses under anesthesia with 1 and 5 μ g of VLPs respectively, while the third group received 5- μ g doses intranasally without anesthesia and the fourth group received 1- μ g doses subcutaneously. Samples of serum, saliva, and vaginal washes were taken 2 weeks after the systemic priming; 2, 4, 6, and 8 weeks after the second immunization; and 2, 4, and 8 weeks after the third immunization (Fig. 5). After a single subcutaneous immunization with a 1- μ g VLP dose, the specific IgG titers measured in the serum were about 10-fold higher than those measured with a single 5- μ g VLP dose administered nasally under anesthesia (compare Fig. 3 and 5), indicating that systemic immunization is indeed more efficient than in-

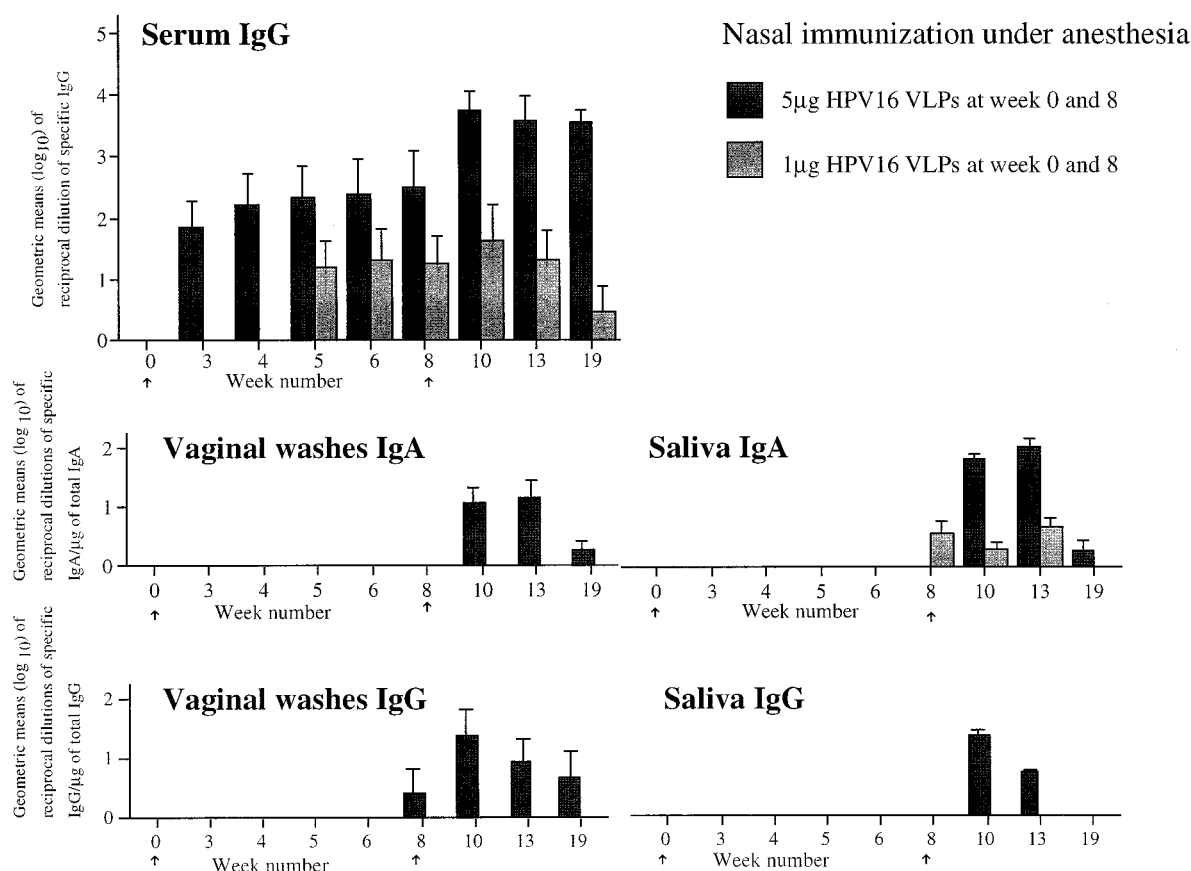


FIG. 3. Anti-HPV16 VLP systemic and mucosal antibody responses after single or double nasal immunizations. Two groups of four 6-week-old BALB/c anesthetized mice were immunized at week 0 and at week 8 with 1 or 5 µg of HPV16 VLP by the intranasal route. Data are expressed as the geometric means (\log_{10}) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or specific IgG per microgram of total IgG in secretions. Error bars indicate the standard errors of the means.

tranasal immunization at inducing IgG responses. After the second immunization, a similar rise in the specific IgG titer in serum was observed in all groups of mice independently of the route of immunization. The low specific IgG titers measured in saliva became undetectable at week 10. Variable but higher specific IgG titers were measured in vaginal washes, and this was seen more consistently in mice given 5-µg booster doses either subcutaneously or intranasally while anesthetized (Fig. 5). As expected, no specific IgA was detected in mice given subcutaneous booster doses whereas significant VLP-specific IgA titers were measured in saliva and vaginal washes in mice given intranasal booster doses. This is in contrast to single or double intranasal immunizations, by either dose regimen or mode, which induced no or barely detectable specific IgA (Fig. 3). The data indicate that systemic priming both overcomes the nonresponsiveness to nasal immunization in conscious mice and enhances overall the efficacy of intranasal immunization. Although the second boost induced some increase in the specific antibody titers in serum and secretions, this effect was transient, except for IgG in saliva, and thus was of little benefit.

To further compare a three-dose protocol of immunization either with or without systemic priming, we immunized three groups of four mice with three weekly VLP doses. The first group received the 5-µg VLP doses intranasally under anesthesia, while the second and third groups were subcutaneously primed with 1 µg of VLP and then given two booster doses with 5 µg of VLPs intranasally under anesthesia or while con-

scious, respectively (Fig. 6). From this direct comparison, it appears that three intranasal 5-µg VLP doses were more effective at inducing specific antibodies in secretion than were the other methods tested in this study.

Mucosal secretions containing HPV16 VLP specific IgA and/or IgG neutralize HPV16 pseudotyped viruses. To assess the neutralization potential of mucosal secretions containing anti-HPV16 VLP antibodies, saliva and genital secretions were tested in the HPV16 pseudovirion in vitro neutralization assay (50) at two different dilutions (Table 1). Serum and secretions obtained by a triple intranasal immunization under anesthesia in the presence and absence of CT (Table 1; Fig. 7), as well as by a triple spaced subcutaneous immunization (Fig. 7), were tested. The absolute titers of VLP-specific IgG and/or IgA in the samples that were used for neutralization experiments (Table 1; Fig. 7) were plotted against their efficiency of neutralization (Fig. 7). In samples containing both specific IgA and IgG, the titers of the two specific isotypes were added, and the total titers are indicated (Fig. 7). The neutralizing activity of the anti-HPV16 VLP monoclonal antibody H16.V5 (isotype IgG2b), was also determined. The data presented were derived from three independent experiments involving an HPV16 pseudovirion input of 22, 44, and 54 focus-forming units. Best-fitting sigmoidal curves were drawn (GraphPad, Prism [Fig. 7]), and the VLP-specific antibody titers at which 50% HPV16 pseudovirion neutralization occurs in vitro were calculated for each group, i.e., 64 for secretions containing both specific IgA

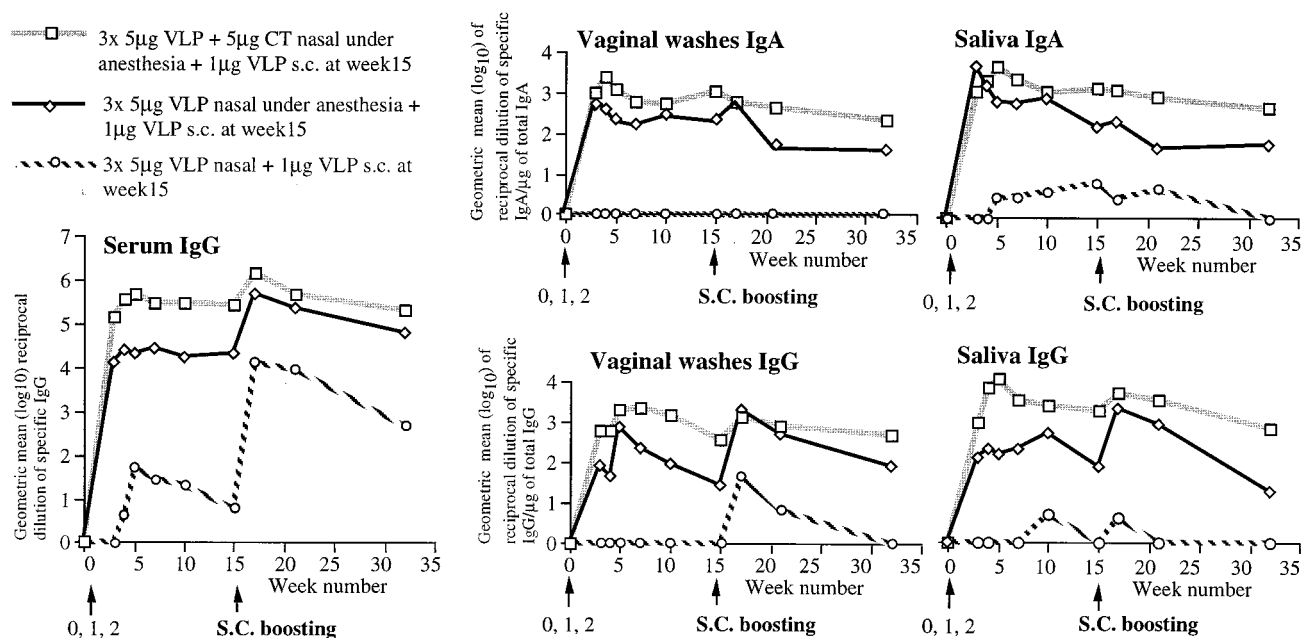


FIG. 4. Anti-HPV16 VLP systemic and mucosal antibody responses following systemic boosting. Three groups of mice previously immunized (Fig. 1 and conscious mice) by the intranasal route, three times weekly, with 5 µg of HPV16 VLP mixed with CT or alone under anesthesia or conscious were given subcutaneous (s.c.) booster doses at week 15 with 1 µg of HPV16 VLP. Data are expressed as the geometric means (log₁₀) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or specific IgG per microgram of total IgG in secretions. Error bars have been deleted to simplify reading.

and IgG, 98 for secretions containing specific IgG alone, 125 for sera (specific IgG alone), and 214 for H16.V5. The values for 50% neutralization by serum and secretions containing specific IgG alone were not statistically different by Tukey's multiple-comparison test ($P > 0.05$). The neutralization efficiency of the VLP-specific IgG from serum and secretions was very similar for individual animals that had been immunized parenterally (data not shown). Examination of the titers of IgA and IgG in the samples in Table 1 suggests that the VLP-specific IgA elicited in secretions of immunized mice is neutralizing, since many samples contain too low an IgG titer to account for the neutralization observed, as judged from the IgG titer determination curve for serum or secretions containing only IgG (Fig. 7). Furthermore, the titer for 50% neutralization by secretions containing both IgA and IgG, 64, was significantly ($P < 0.05$) lower than for serum (titer of specific IgG, 125) and was lower than for secretions containing specific IgG alone (titer, 98), although not significantly so.

DISCUSSION

Infection by genital HPV is believed to occur when minor trauma expose the basal cells of the genital squamous epithelium to virus. Sterilizing immunity therefore would be mediated by neutralizing antibodies located in the vaginocervical secretion or close to the basal cells at the time of infection. Furthermore, neutralizing antibody in mucosal secretions could limit transmission of breakthrough infection. A prophylactic vaccine against genital HPVs should therefore be capable of inducing HPV neutralizing antibodies in genital secretions throughout the menstrual cycle and over long periods. It might also be advantageous to produce neutralizing antibodies in other mucosal sites of HPV infection, i.e., oral and anal sites, in which HPV-associated cancers are known to occur. Systemic immunizations induce only transudated antibodies, mainly IgG, from the serum in genital secretions (8, 45), while

mucosal immunizations can induce both transudated IgG and locally produced, mainly sIgA, antibodies (9, 39). However, the induction of a strong and/or protective immune response in the genital tract is highly variable and depends on the nature of the immunogen, the addition of mucosal adjuvants, the route of immunization, and the host species (63).

In this study, we have measured the antibody response induced in serum as well as in oral and genital secretions of mice by intranasal immunization with purified HPV16 VLPs. Our data demonstrate that low doses of the VLP antigen, probably due to its particulate and repetitive, closely packed antigenic characteristics (2), are highly immunogenic when delivered by the intranasal route even in the absence of the mucosal adjuvant CT. The same doses induced only barely detectable specific antibodies via the oral route, probably due to antigen degradation in the stomach and intestine. However, the doses used were 50- to 1,000-fold lower than the amounts of soluble antigen previously used successfully to induce antibody responses via the oral route (26, 36). It is therefore possible that high doses of VLPs will be capable of generating a substantial response after oral delivery, as has been shown with rotavirus VLPs (46). The nasal route also appeared to be the most efficient route of immunization for other antigens (1, 14, 16, 17, 20, 25, 32, 37, 43, 46, 47, 52, 55, 61). Immunization by a triple 5-µg intranasal dose of VLP was more efficient (Fig. 1) than systemic immunization by a triple, spaced, 1-µg VLP dose (Fig. 5), since it induced similar titers of specific IgG in secretions but also induced specific sIgA. However, our data suggest that the VLP antigen should contact the lungs to induce an effective antibody response in mice. If safe, immunization via the lungs in humans could be achieved with aerosols.

The mechanisms by which intranasal immunization induces an immune response are not fully understood. Nasal-associated lymphoid tissue (NALT) appears to play a key role in rodents (65), but the contributions of the bronchus-associated lymphoid tissue (BALT [54]) and/or intraepithelial dendritic

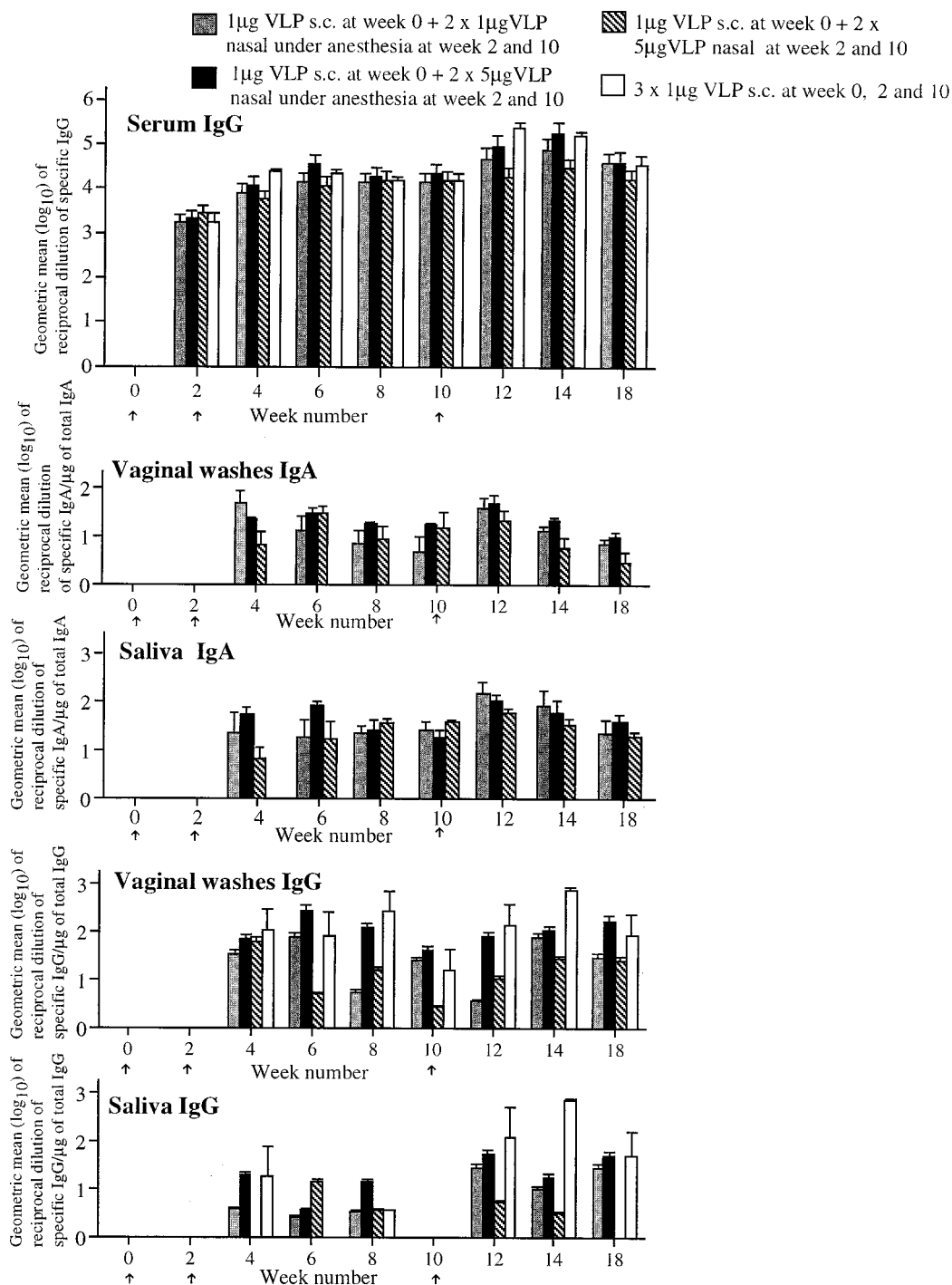


FIG. 5. Anti-HPV16 VLP systemic and mucosal antibody responses in mice systemically primed. Four groups of four 6-week-old BALB/c mice were primed with 1 μ g of HPV16 VLP subcutaneously and then given booster doses at weeks 2 and 10 either intranasally with 1 or 5 μ g of HPV16 VLP under anesthesia or conscious or subcutaneously (s.c.) with 1 μ g of HPV16 VLP. Data are expressed as the geometric means (\log_{10}) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or specific IgG per microgram of total IgG in secretions. Error bars indicate the standard errors of the means.

cells are unclear. In our experimental system, BALT appears to be crucial for generating an effective antibody response and NALT is less effective. Intranasal immunization of conscious mice, i.e., via NALT, does not induce immune tolerance or suppression (60), since strong specific antibody responses could be induced by a subsequent systemic boost (Fig. 4).

Our data obtained with mice suggest that the nonresponse after intranasal immunization in the absence of anesthesia may be overcome by systemic priming followed by an intranasal boost. Systemic immunization with microencapsulated antigens has previously been shown to prime for the induction of disseminated mucosal IgA responses by subsequent mucosal

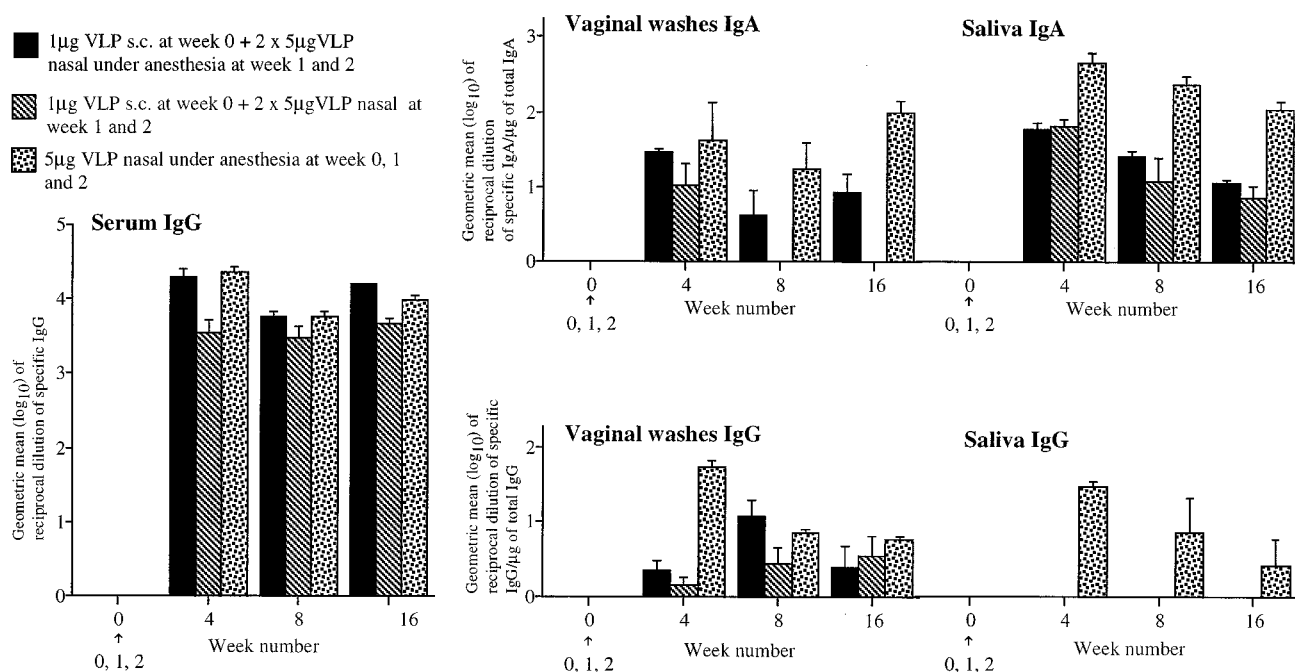


FIG. 6. Direct comparison of anti-HPV16 VLP systemic and mucosal antibody responses in mice systemically primed or only intranasally immunized. Three groups of four 6-week-old BALB/c mice were either primed subcutaneously with 1 µg of HPV16 VLP and then given intranasal booster doses of 5 µg at weeks 1 and 2 under anesthesia or conscious or only intranasally immunized three times weekly with 5 µg of HPV16 VLP under anesthesia. Data are expressed as the geometric means (\log_{10}) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or IgG per microgram of total IgG in secretions. Error bars indicate the standard errors of the means.

boosting by either the oral or intratracheal route in mice (18) and in monkeys (37). It is, however, unclear how the systemic and mucosal immune systems interact. Antigen delivery to the MALT leads to a generalized secretory immune response. After antigen processing and presentation by dendritic cells, committed B lymphocytes proliferate in the MALT and then migrate via the lymph and eventually reach all secretory tissues. This results in the ultimate appearance of antigen-specific sIgA in all of the mucosal secretions (6, 9), including the female genital secretions, as predicted by the concept of a common mucosal system (39, 40). The homing of mucosally primed immunoblasts to mucosal tissue is mediated by specific homing receptors ($\alpha 4\beta 7$ [4, 23]) that recognize their counterparts, mu-

cosal addressins (MAdCAM-1 [4, 11]), in the high endothelial venules of the gut. The mucosal addressins responsible for homing to nonintestinal mucosal sites such as the salivary gland or the genital mucosa are not known. In contrast, immunoblasts primed in peripheral lymph nodes (PLN) bear L-selectin homing receptor and home to PLN (3). Interestingly, intranasally primed human immunoblasts have been shown to coexpress mucosal and systemic homing receptors (49), suggesting that these lymphocytes might home to both mucosal sites and PLN, where interactions with systemically primed lymphocytes could occur.

sIgA antibodies are believed to be the primary effectors of mucosal immunity, and their superiority over IgG in immune

TABLE 1. Analysis of saliva and vaginal washes obtained from mice immunized three times intranasally while anesthetized by using 5 µg of HPV16 VLPs, either with or without 5 µg of CT

Source/sample	CT adjuvant used	Final sample dilution	Anti-HPV16 VLP IgA titers	Anti-HPV16 VLP IgG titers	Neutralization (%)
Vaginal wash/A	+	3.3×10^{-1}	591	197	100
Vaginal wash/A	+	4.9×10^{-2}	88	30	100
Vaginal wash/B	+	2.1×10^{-1}	103	27	100
Vaginal wash/B	+	2.6×10^{-2}	13	3	46
Vaginal wash/C		3×10^{-1}	91	6	91
Vaginal wash/C		4.4×10^{-2}	13	1	0
Vaginal wash/D	+	9×10^{-2}	18	7	43
Vaginal wash/D	+	1×10^{-2}	2	0.8	0
Vaginal wash/E		1.3×10^{-1}	26	1.3	22
Vaginal wash/E		1.5×10^{-2}	3	0.2	0
Saliva/F	+	9×10^{-2}	169	12	100
Saliva/F	+	1×10^{-2}	19	1.3	22
Saliva/G	+	1.1×10^{-1}	89	8	100
Saliva/G	+	1.2×10^{-2}	10	0.8	36
Salvia/H		4×10^{-2}	11	0.4	0

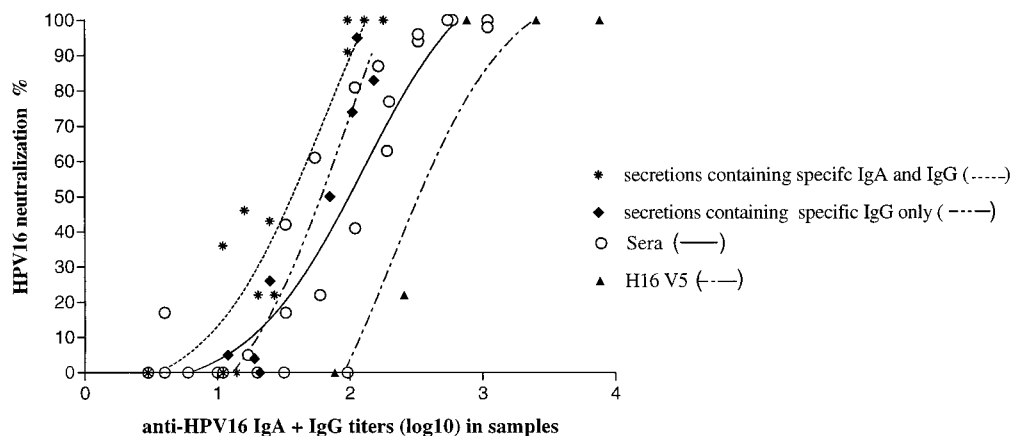


FIG. 7. Neutralization efficacy of anti-HPV16 IgA and/or IgG in secretion and serum. The final titers of anti-HPV16 VLP IgA and/or IgG in the samples that were used for neutralization experiments were plotted against the neutralization efficacy elicited. Best-fitting sigmoidal curves were drawn with the different groups (GraphPad, Prism). In the secretions that contained both specific IgA and IgG (Table 1), the titers of the two specific isotypes were added and the summed value is indicated.

defense mechanisms has often been suggested (5, 13). Resistance to mucosal infection has been strongly correlated both with the presence of specific IgA in mucosal secretions (41, 44) and with the number of IgA-secreting cells at the site of infection (66). Although systemic passive transfer of immune serum IgG protected animals from experimental papillomavirus infection, even at oral sites, the ability of a systemic IgG response and the importance of sIgA in protecting from natural genital transmission remain unknown. Induction of both antibody classes may be important to achieve continuous protection, since it has been shown that both IgA and IgG levels in genital secretions vary greatly and inversely during the estrous cycle (19, 31, 59, 62). Low levels of transudated IgG and partial in vitro neutralization by anti-VLP IgG alone in genital secretions of monkeys immunized parenterally with HPV11 VLPs have been demonstrated (33).

Our data provide an opportunity to compare the efficacy of in vitro neutralization of pseudotype HPV16 by antibodies in saliva and genital secretions with those in serum. The neutralizing activities of the polyclonal IgG antibody samples were not statistically different ($P > 0.05$ by Tukey's multiple-comparison test), regardless of whether the antibody was secreted or serum derived (50% neutralization at titers of 98 and 125, respectively). Since the secreted IgG of mice immunized parenterally is thought to be primarily the result of transudation from the serum, its properties would be expected to match those of serum IgG.

Several reports indicate that the neutralizing efficacy of IgA antibodies may be greater than that of IgG (5, 35, 42), and 50% neutralization was obtained by secretions containing both IgG and IgA with a titer of 64, yet titers of 98 (not significantly different) and 125 ($P < 0.05$ by Tukey's multiple-comparison test) were required by secretions containing only IgG and serum, respectively (Fig. 7). However, the ratios of IgA and IgG varied between secretions (Table 1), and there is potential for subtly different ELISA sensitivities for each antibody isotype. Study of HPV16 pseudovirion neutralization by purified antibodies of each isotype is required to determine differential efficacy of neutralization by IgG and IgA.

Determination of the titers of VLP-specific mouse antibodies demonstrated similar activity to the anti-HPV11 virion sera derived from rabbits and African green monkeys tested (12, 33) by using the athymic mouse xenograft assay for HPV11

neutralization. This suggests that the two in vitro neutralization assays have similar sensitivities and that monkeys, rabbits, and mice produce VLP-specific antibodies of quite similar neutralizing efficacy regardless of whether the antigen was purified from warts, insect cells, or yeast. Further, the VLP ELISA data correlated well with the in vitro neutralization data in these studies, implying that the VLP ELISA is a good surrogate assay for in vitro neutralization.

Although caution must be used in extrapolating our observations to humans, due to substantial differences in the anatomy of the nasal passages and the associated lymphoid tissues, our findings raise the possibility that nasal immunization with purified HPV VLPs may be an effective and well-tolerated method for inducing both HPV-neutralizing IgA and IgG in the genital secretions of women.

ACKNOWLEDGMENTS

We thank Jean-Pierre Kraehenbuhl and Douglas Lowy for critical reading of the manuscript.

This work was supported by Fonds de Service of the Dept. of Gynecology and Swiss National Funds 31-52892.97 to D.N.-H. and by the intramural program of the National Cancer Institute.

REFERENCES

- Allardyce, R. A. 1984. Effect of ingested sperm on fecundity in the rat. *J. Exp. Med.* **159**:1548-1553.
- Bachmann, M. F., and R. M. Zinkernagel. 1996. The influence of virus structure on antibody responses and virus serotype formation. *Immunol. Today* **17**:553-558.
- Berg, E. L., M. K. Robinson, R. A. Warnock, and E. C. Butcher. 1991. The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J. Cell Biol.* **114**:343-349.
- Berlin, C., E. L. Berg, M. J. Briskin, D. P. Andrew, P. J. Kilshaw, B. Holzmann, I. L. Weissman, A. Hamann, and E. C. Butcher. 1993. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* **74**:185-195.
- Bessen, D., and V. A. Fischetti. 1988. Passive acquired mucosal immunity to group A streptococci by secretory immunoglobulin A. *J. Exp. Med.* **167**:1945-1950.
- Bienenstock, J., M. McDermott, D. Befus, and M. O'Neill. 1978. A common mucosal immunologic system involving the bronchus, breast and bowel. *Adv. Exp. Med. Biol.* **107**:53-59.
- Bosch, F. X., M. M. Manos, N. Munoz, M. Sherman, A. M. Jansen, J. Peto, M. H. Schiffman, V. Moreno, R. Kurman, and K. V. Shah. 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International Biological Study on Cervical Cancer (IBSCC) Study Group. *J. Natl. Cancer Inst.* **87**:796-802.

8. Bouvet, J. P., L. Belec, R. Pires, and J. Pillot. 1994. Immunoglobulin G antibodies in human vaginal secretions after parenteral vaccination. *Infect. Imm.* **62**:3957–3961.
9. Brandtzaeg, P. 1989. Overview of the mucosal immune system. *Curr. Top. Microbiol. Immunol.* **146**:13–25.
10. Breitburd, F., R. Kirnabauer, N. Hubbert, B. Nonnenmacher, C. Trin-Dinh-Desmarquet, G. Orth, J. T. Schiller, and D. R. Lowy. 1995. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J. Virol.* **69**:3959–3963.
11. Briskin, M., D. Winsorhines, A. Shyjan, N. Cochran, S. Bloom, J. Wilson, L. M. Mcevoy, E. C. Butcher, N. Kassam, C. R. Mackay, W. Newman, and D. J. Ringler. 1997. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. *Am. J. Pathol.* **151**:97–110.
12. Bryan, J. T., K. U. Jansen, R. S. Lowe, K. H. Fife, T. McClowry, D. Glass, and D. R. Brown. 1997. Human papillomavirus type 11 neutralization in the athymic mouse xenograft system—correlation with virus-like particle IgG concentration. *J. Med. Virol.* **53**:185–188.
13. Childers, N. K., M. G. Bruce, and J. R. McGhee. 1989. Molecular mechanisms of immunoglobulin A defense. *Annu. Rev. Microbiol.* **43**:503–536.
14. Dehaan, A., K. B. Renegar, P. J. Small, and J. Wilschut. 1995. Induction of a secretory IgA response in the murine female urogenital tract by immunization of the lungs with liposome-supplemented viral subunit antigen. *Vaccine* **13**:613–616.
15. Dehaan, A., J. F. Tomee, J. P. Huchshorn, and J. Wilschut. 1995. Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice. *Vaccine* **13**:1320–1324.
16. Di Tomaso, A., G. Saletti, M. Pizzo, R. Rappuoli, G. Dougan, S. Abrignani, G. Douce, and M. M. De. 1996. Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. *Infect. Imm.* **64**:974–979.
17. Drew, M. D., C. A. Estrada, B. J. Underdown, and M. R. McDermott. 1992. Vaccination by cholera toxin conjugated to a herpes simplex virus type 2 glycoprotein D peptide. *J. Gen. Virol.* **73**:2357–2366.
18. Eldridge, J. H., J. K. Staas, J. A. Meulbroek, J. R. McGhee, T. R. Tice, and R. M. Gilley. 1991. Biodegradable microspheres as a vaccine delivery system. *Mol. Immunol.* **28**:287–294.
19. Gallichan, W. S., and K. L. Rosenthal. 1996. Effects of the estrous cycle on local humoral immune responses and protection of intranasally immunized female mice against herpes simplex virus type 2 infection in the genital tract. *Virology* **224**:487–497.
20. Gallichan, W. S., and K. L. Rosenthal. 1995. Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus. *Vaccine* **13**:1589–1595.
21. Hagensee, M. E., J. J. Carter, G. C. Wipf, and D. A. Galloway. 1995. Immunization of mice with HPV vaccinia virus recombinants generates serum IgG, IgM, and mucosal IgA antibodies. *Virology* **206**:174–182.
22. Hagensee, M. E., N. Yaegashi, and D. A. Galloway. 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *J. Virol.* **67**:315–322.
23. Hamann, A., D. P. Andrew, W. D. Jablonski, B. Holzmans, and E. C. Butcher. 1994. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J. Immunol.* **152**:3282–3293.
24. Hofmann, K. J., J. C. Cook, J. G. Joyce, D. R. Brown, L. D. Schultz, H. A. George, M. Rosolowsky, K. H. Fife, and K. U. Jansen. 1995. Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. *Virology* **209**:506–518.
25. Hopkins, S., J.-P. Kraehenbuhl, F. Schödel, A. Potts, D. Peterson, P. De Grandi, and D. Nardelli-Haeffiger. 1995. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. *Infect. Imm.* **63**:3279–3286.
26. Jackson, R. J., K. Fujihashi, A. J. Xu, H. Kiyono, C. O. Elson, and J. R. McGhee. 1993. Optimizing oral vaccines: induction of systemic and mucosal B-cell and antibody responses to tetanus toxin by use of cholera toxin as an adjuvant. *Infect. Imm.* **61**:4272–4279.
27. Johansson, E. L., C. Rask, M. Fredriksson, K. Eriksson, C. Czerkinsky, and J. Holmgren. 1998. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin b subunit or conjugates. *Infect. Imm.* **66**:514–520.
28. Kirnbauer, R., F. Booy, N. Cheng, D. R. Lowy, and J. T. Schiller. 1992. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* **89**:12180–12184.
29. Kirnbauer, R., L. M. Chandrachud, B. W. Oneil, E. R. Wagner, G. J. Grindlay, A. Armstrong, G. M. Megarvie, J. T. Schiller, D. R. Lowy, and M. S. Campo. 1996. Virus-like particles of bovine papillomavirus type 4 in prophylactic and therapeutic immunization. *Virology* **219**:37–44.
30. Kirnbauer, R., J. Taub, H. Greenstone, R. Roden, M. Dürst, L. Gissmann, D. R. Lowy, and J. T. Schiller. 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *J. Virol.* **67**:6929–6936.
31. Kutteh, W. H., R. E. Blackwell, H. Gore, C. C. Kutteh, B. R. Carr, and J. Mestecky. 1990. Secretory immune system of the female reproductive tract. II. Local immune system in normal and infected fallopian tube. *Fertil. Steril.* **54**:51–55.
32. Lehner, T., C. Panagiotidi, L. A. Bergmeier, T. Ping, R. Brookes, and A. S. E. 1992. A comparison of the immune response following oral, vaginal, or rectal route of immunization with SIV antigens in nonhuman primates. *Vaccine Res.* **1**:319–330.
33. Lowe, R. S., D. R. Brown, J. T. Bryan, J. C. Cook, H. A. George, K. J. Hofmann, W. M. Hurni, J. G. Joyce, E. D. Lehman, H. Z. Markus, M. P. Neep, L. D. Schultz, A. R. Shaw, and K. U. Jansen. 1997. Human papillomavirus type 11 (hpv-11) neutralizing antibodies in the serum and genital mucosal secretions of african green monkeys immunized with hpv-11 virus-like particles expressed in yeast. *J. Infect. Dis.* **176**:1141–1145.
34. Lowy, D. R., R. Kirnbauer, and J. T. Schiller. 1994. Genital human papillomavirus infection. *Proc. Natl. Acad. Sci. USA* **91**:2436–2440.
35. Magnusson, K. E., and I. Stjernstrom. 1982. Mucosal barrier mechanisms. Interplay between secretory IgA (SIgA), IgG and mucins on the surface properties and association of salmonellae with intestine and granulocytes. *Immunology* **45**:239–248.
36. Marinaro, M., H. F. Staats, T. Hiroi, R. J. Jackson, M. Coste, P. N. Boyaka, N. Okahashi, M. Yamamoto, H. Kiyono, H. Bluethmann, et al. 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol.* **155**:4621–4629.
37. Marx, P. A., R. W. Compans, A. Gettie, J. K. Staas, R. M. Gilley, M. J. Mulligan, G. V. Yamschikov, D. Chen, and J. H. Eldridge. 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. *Science* **260**:1323–1327.
38. Marx, P. A., R. W. Compans, A. Gettie, J. K. Staas, R. M. Gilley, M. J. Mulligan, G. V. Yamschikov, D. Chen, and J. H. Eldridge. 1993. Protection against vaginal SIV transmission with microencapsulated vaccine. *Science* **260**:1323–1327.
39. McDermott, M., and J. Bienenstock. 1979. Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. *J. Immunol.* **122**:1892–1898.
40. McDermott, M. R., D. A. Clark, and J. Bienenstock. 1980. Evidence for a common mucosal immunologic system. II. Influence of the estrous cycle on B immunoblast migration into genital and intestinal tissues. *J. Immunol.* **124**:2536–2539.
41. Mills, J. T., K. J. Van, P. F. Wright, and R. M. Chanock. 1971. Experimental respiratory syncytial virus infection of adults. Possible mechanisms of resistance to infection and illness. *J. Immunol.* **107**:123–130.
42. Mogens, D., and M. W. Russell. 1994. Function of mucosal immunoglobulins, p. 127–137. *In* P. L. Gra et al. (ed.), *Handbook of mucosal immunology*. Academic Press, Inc., San Diego, Calif.
43. Nardellihaefliger, D., R. Roden, J. Benyacoub, R. Sahli, J. P. Kraehenbuhl, J. T. Schiller, P. Lachat, A. Potts, and P. Degrandi. 1997. Human papillomavirus type 16 virus-like particles expressed in attenuated salmonella typhimurium elicit mucosal and systemic neutralizing antibodies in mice. *Infect. Imm.* **65**:3328–3336.
44. Ogra, P. L., and D. T. Karzon. 1969. Poliovirus antibody response in serum and nasal secretions following intranasal inoculation with inactivated poliovaccine. *J. Immunol.* **102**:15–23.
45. Ogra, P. L., and S. S. Ogra. 1973. Local antibody response to poliovaccine in the human female genital tract. *J. Immunol.* **110**:1307–1311.
46. O'Neal, C. M., S. E. Crawford, M. K. Estes, and M. E. Conner. 1997. Rotavirus virus-like particles administered mucosally induce protective immunity. *J. Virol.* **71**:8707–8717.
47. Pal, S., E. M. Peterson, and L. de la Maza. 1996. Intranasal immunization induces long-term protection in mice against a *Chlamydia trachomatis* genital challenge. *Infect. Imm.* **64**:5341–5348.
48. Parr, M. B., and E. L. Parr. 1985. Immunohistochemical localization of immunoglobulins A, G and M in the mouse female genital tract. *J. Reprod. Fertil.* **74**:361–370.
49. Quidingjarbrink, M., I. Nordstrom, G. Granstrom, A. Kilander, M. Jertborn, E. C. Butcher, A. I. Lazarovits, J. Holmgren, and C. Czerkinsky. 1997. Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations—a molecular basis for the compartmentalization of effector b cell responses. *J. Clin. Invest.* **99**:1281–1286.
50. Roden, R. B. S., H. L. Greenstone, R. Kirnbauer, J. P. Booy, J. Jessie, D. R. Lowy, and J. T. Schiller. 1996. In vitro generation and type-specific neutralization of a human papillomavirus type 16 virion pseudotype. *J. Virol.* **70**:5875–5883.
51. Rose, R. C., R. C. Reichmann, and W. Bonnez. 1994. Human papillomavirus (HPV) type 11 recombinant virus-like particles induce the formation of neutralizing antibodies and detect HPV-specific antibodies in human sera. *J. Gen. Virol.* **75**:2075–2079.
52. Russell, M. W., Z. Moldoveanu, P. L. White, G. J. Sibert, J. Mestecky, and S. M. Michalek. 1996. Salivary, nasal, genital, and systemic antibody re-

- sponses in monkeys immunized intranasally with a bacterial protein antigen and the cholera toxin b subunit. *Infect. Immun.* **64**:1272–1283.
53. **Sasagawa, T., P. Pushko, G. Steers, S. E. Gshmeissner, M. A. N. Hajibaghieri, J. Finch, L. Crawford, and M. Tommasino.** 1995. Synthesis and assembly of virus-like particles of human papillomaviruses type 6 and type 16 in fission yeast *Schizosaccharomyces pombe*. *Virology* **206**:126–135.
 54. **Sminia, T., G. J. van der Brugge-Gamelkoorn, and S. H. Jeurissen.** 1989. Structure and function of bronchus-associated lymphoid tissue (BALT). *Crit. Rev. Immunol.* **9**:119–145.
 55. **Staats, H. F., W. G. Nichols, and T. J. Palker.** 1996. Mucosal immunity to HIV-1: systemic and vaginal antibody responses after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN(A). *J. Immunol.* **157**:462–472.
 56. **Suzich, J. A., S. J. Ghim, F. J. Palmerhill, W. I. White, J. K. Tamura, J. A. Bell, J. A. Newsome, A. B. Jenson, and R. Schlegel.** 1995. Systemic immunization with papillomavirus 11 protein completely prevents the development of viral mucosal papillomas. *Proc. Natl. Acad. Sci. USA* **92**:11553–11557.
 57. **Svennerholm, A. M., L. A. Hanson, J. Holmgren, B. S. Lindblad, B. Nilsson, and F. Quereshi.** 1980. Different secretory immunoglobulin A antibody responses to cholera vaccination in Swedish and Pakistani women. *Infect. Immun.* **30**:427–430.
 58. **Thapar, M. A., E. L. Parr, and M. B. Parr.** 1990. Secretory immune responses in mouse vaginal fluid after pelvic, parenteral or vaginal immunization. *Immunology* **70**:121–125.
 59. **Usala, S. J., F. O. Usala, R. Haciski, J. A. Holt, and G. F. Schumacher.** 1989. IgG and IgA content of vaginal fluid during the menstrual cycle. *J. Reprod. Med.* **34**:292–294.
 60. **Waldo, F. B., A. W. van den Wall Bake, J. Mestecky, and S. Husby.** 1994. Suppression of the immune response by nasal immunization. *Clin. Immunol. Immunopathol.* **72**:30–34.
 61. **Ward, M. E.** 1992. Vaccination against chlamydial genital tract infection: a key problem of mucosal immunology, p. 409–425. *In* P. D. Gufio and P. Johnson (ed.), *Local immunity in reproductive tract tissues*. Oxford University Press, New Delhi, India.
 62. **Wira, C., J. Richardson, and R. Pabhala.** 1994. Endocrine regulation of mucosal immunity: effect of sex hormones and cytokine on the afferent and efferent arms of the immune system in the female reproductive tract. Academic press, Inc., San Diego, Calif.
 63. **Wira, C. R., and C. Kaushic.** 1996. Mucosal immunity in the female reproductive tract: effect of sex hormones on immune recognition and responses, p. 375–388. *In* H. Kiyono, P. L. Ogra, and J. R. McGhee (ed.), *Mucosal vaccines*. Academic Press, Inc., San Diego, Calif.
 64. **Wira, C. R., and C. P. Sandoe.** 1987. Origin of IgA and IgG antibodies in the female reproductive tract: regulation of the genital response by estradiol. *Adv. Exp. Med. Biol.* **216A**:403–412.
 65. **Wu, H. Y., and M. W. Russell.** 1997. Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system. *Immunol. Res.* **16**:187–201.
 66. **Yuan, L., L. A. Ward, B. I. Rosen, T. L. To, and L. J. Saif.** 1996. Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J. Virol.* **70**:3075–3083.